Duke NAFLD Clinical Research Program

IRB Protocol Summary
Confidential

Impact of Fructose on Metabolism, Energy Homeostasis and MR biomarkers in NAFLD

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1. Hypothesis and Principal Objective

1.1 Primary hypothesis

- Fructose is a risk factor for hepatic steatosis, NASH, and advanced fibrosis and that fructose exposure will worsen the metabolic, energy homeostatic and associated MR based biomarker associated features of nonalcoholic fatty liver disease (NAFLD).

1.2 Principal objectives:

- To define whether baseline and dynamic changes in hepatic ATP during a fructose challenge are associated with NAFLD and/or liver disease severity in patients with NAFLD.
- To assess whether such factors can be measured using magnetic resonance (MR) biomarkers

We will measure *baseline and dynamic* changes in metabolism, energy homeostasis and MR biomarkers in healthy volunteers and patients with biopsy-proven NAFLD. The effect of acute fructose challenges on changes in metabolism and energy homeostasis will be assessed in a cohort of patients with NAFLD.

2. Background and Significance

2.1 Prevalence of NAFLD

Nonalcoholic fatty liver disease (NAFLD) is now the most common liver disease in the United States and possibly worldwide (1). Nonalcoholic steatohepatitis (NASH), a more serious form of NAFLD, can progress to cirrhosis and even hepatocellular carcinoma (2). These liver diseases represent the hepatic component of the metabolic syndrome (3). The epidemic of NAFLD-related chronic liver disease has paralleled the rise in obesity world-wide.

2.2 Fructose as a Risk Factor for NAFLD

Like obesity, NAFLD and NASH are closely linked to nutrition and the "Western diet" which is rich in saturated fats and refined sugars. Although fat consumption has remained relatively stable, the marked increase in dietary fructose consumption (more than doubling in the past 30 years alone) supports the role of fructose in NAFLD and the metabolic syndrome (4-6). Although the mechanism(s) for fructose-related liver injury is not yet well defined, fructose-related hepatic adenosine triphosphate (ATP) depletion may contribute to liver injury. Observations in animals suggest that fructose induces metabolic syndrome and NAFLD independent of energy intake (7, 8). One key difference in fructose metabolism (as opposed to glucose) relates to ATP depletion and the necessity of adenosine monophosphate (AMP) kinase to replenish ATP stores. As opposed to glucose, initial fructose metabolism involves phosphorylation of fructose to fructose-1-phosphate by fructokinase (ketohexokinase, KHK) using the substrate ATP. Unlike glucokinase, the phosphorylation of fructose by KHK is specific for fructose and not rate limited (9). Replenishment of ATP stores requires phosphorylation of AMP back to ATP via AMP kinase (which is inhibited in insulin resistance (common *in patients with NAFLD) or conversion to uric acid via xanthine dehydrogenase resulting in* hyperuricemia.

The high activity of KHK in phosphorylating fructose to fructose-1-phosphate in the liver, could result in hepatic ATP depletion (10) with habitual fructose consumption.

Published animal and human studies support our hypothesis that fructose is a risk factor for NAFLD and NAFLD-related liver disease progression. In animal models, diets high in fructose induce features of the metabolic syndrome including weight gain, insulin resistance, hypertriglyceridemia, and hypertension (11). Similar effects are not observed with the administration of other simple sugars such as glucose (8). Fructose (or sucrose) administration to humans also causes features of metabolic syndrome (10, 12, 13) which are quite typical of patients with NAFLD. Fructose is lipogenic, stimulates triglyceride synthesis (14) and causes hepatic steatosis (15, 16). As previously reported in animals, our group reported that increased fructose consumption (assessed as fructose-containing beverages only) is a risk factor of metabolic syndrome and biopsy-proven NAFLD and that patients with NAFLD consume 3-4 times more fructose than age, gender, and mass index (BMI) matched controls without liver disease (17).

In addition to increased fructose consumption being a risk factor for NAFLD (17), *fructose has been implicated in NAFLD disease progression* (18, 19). The administration of a diet with 25% of total energy as sucrose (which contains 50% fructose) resulted in a rise in liver aminotransferase levels within 18 days (18). This study, performed nearly 25 years ago, is all the more alarming as current sugar intake of Americans is in this same range (4, 5). In our study of 427 patients with biopsy-proven NAFLD, increased consumption of fructose-containing beverages was univariately associated with decreased age ($P < 0.0001$), male sex ($P < 0.0001$), hypertriglyceridemia ($P < 0.04$), low high density lipoprotein (HDL) cholesterol (<0.0001), decreased serum glucose ($P < 0.001$), increased calorie intake ($P < 0.0001$), and hyperuricemia ($P < 0.0001$) (19). After controlling for age, sex, BMI, and total calorie intake, daily fructose consumption was associated with *lower steatosis* grade and *higher fibrosis* stage ($P < 0.05$ for each). Being that triglyceride synthesis requires ATP, we hypothesize that lower hepatic steatosis may reflect deceased ATP availability. Additionally, in older adults (age $\geq$ 48 years), daily fructose consumption was associated with increased hepatic inflammation ($P < 0.05$), and hepatocyte ballooning ($P< 0.05$) (19). However, the *mechanism(s) by which fructose causes liver injury remains unknown.*

2.3 Fructose Alters Energy Homoeostasis and Causes Hyperuricemia

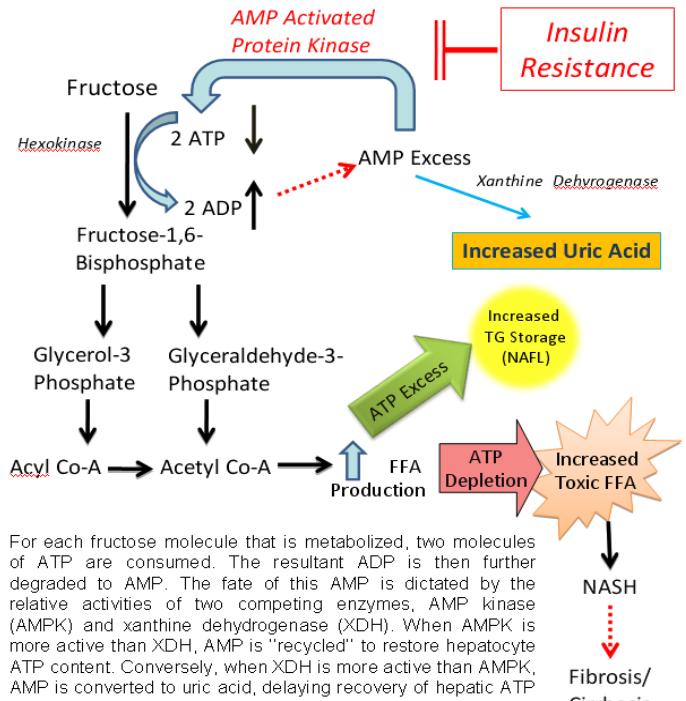
In support of our hypothesis that ATP depletion underlies liver injury in patients with NAFLD, our group has demonstrated that patients with biopsy-proven NAFLD have increased hepatic mRNA expression of KHK compared to matched controls (17). Indeed, in human pilot studies, intravenous (IV) fructose administration is associated with hepatic ATP depletion (20, 21) which can be assessed by ^{31}P magnetic resonance spectroscopy (MRS) (22). Reduced hepatic ATP stores are more prevalent in overweight and obese subjects than in lean subjects (23). Furthermore, recovery from fructose-induced ATP depletion was found to be delayed in patients with NAFLD (n=8) (24). However, a limitation to this existing work is the small sample size and the inability to assess a cause-effect relationship(s) between BMI, NAFLD, energy homeostasis, and histologic features of liver injury. In liver cells, ATP depletion could perpetuate chronic liver injury by making fatty hepatocytes less proliferative. Hepatic ATP depletion also encourages the expansion of liver progenitor populations (25), causes arrest in protein synthesis, induces inflammatory and prooxidative changes (26, 27), increases endoplasmic reticulum stress, promotes activation of stress-related kinases, induces mitochondrial dysfunction, and increases

apoptotic activity (28-30). This supporting data suggests that fructose may be associated with NAFLD, NASH, and progressive fibrosis. Further, a study by Loguercio *et al.* demonstrated that increased uric acid levels above the basal level after IV fructose infusion was significantly higher ($p < 0.01$) in patients with cirrhosis (3 mg/dl) and NASH (1.9 mg/dl) than in healthy controls (1.2 mg/dl) (31). This effect was completely reversed by fructose 1,6-diphosphate which could replenish the ability to resynthesize ATP from ADP. Therefore, an IV fructose challenge could effectively differentiate healthy subjects, from chronic hepatitis, from cirrhosis (31).

2.4 Hepatic ATP Depletion and Hyperuciemia as Potential Non-invasive Biomarkers for NAFLD

NAFLD lacks accurate and robust non-invasive biomarkers to grade and stage histologic disease activity. This is a critical barrier to understanding the influence of this important environmental risk factor (increased/habitual fructose consumption) on the pathogenesis and progression of NAFLD. Currently, reliable assessment NAFLD requires liver biopsy and interpretation of histology. Serum aminotransferase levels and conventional imaging methods can detect liver fat but cannot grade or stage NAFLD (32-35). Furthermore, current developments in biomarker are cross-sectional in nature and do not characterize the *dynamic* changes which underlie liver injury in patients with NAFLD. *In vivo* ^{31}P MRS permits the evaluation of *dynamic changes* of individual phosphorus-containing metabolites in the liver parenchyma, such as phosphomonoester (PME), ATP, and inorganic phosphate (Pi). Intravenous fructose load alters phosphorus metabolites and allows assessment of liver function by ^{31}P MRS (36). Other investigators have demonstrated that fructose loading could be used effectively as a tool to investigate change in metabolic steps of hepatic metabolism in humans with alcohol-related liver disease (36). Further, IV fructose loading causes significantly higher ATP degradation and uric acid production in cirrhotic patients than in healthy controls (36). The associations between fructose, increased uric acid, and hepatic ATP depletion has been previously described (14, 17, 19, 31, 36). Increased uric acid is an independent risk factor for NAFLD (37-41) and in keeping with our hypothesis, hyperuricemia may be a surrogate marker of impaired hepatic energy homeostasis in patients with NAFLD. The proposed mechanism for fructose-related hepatic ATP depletion, NAFLD, NASH and the associated hyperuricemia is depicted in Figure 1 is *novel, innovative, scientifically rigorous and address an important public health concern*—the impact of fructose on the rising epidemic of NAFLD.

Figure 1.
Fructose Associated Hepatic ATP Depletion



For each fructose molecule that is metabolized, two molecules of ATP are consumed. The resultant ADP is then further degraded to AMP. The fate of this AMP is dictated by the relative activities of two competing enzymes, AMP kinase (AMPK) and xanthine dehydrogenase (XDH). When AMPK is more active than XDH, AMP is "recycled" to restore hepatocyte ATP content. Conversely, when XDH is more active than AMPK, AMP is converted to uric acid, delaying recovery of hepatic ATP stores. Insulin resistance, which decreases AMPK activity, further augments the effect of fructose metabolism, resulting in hepatic ATP depletion.

2.5 Use of MRI/ MRS as a Non-invasive Biomarker of NAFLD

Various MRI/MRS methods are used to investigate liver status (42-45). Tools used in this study will be extensions of these methods validated in other organs, and that we have adapted for use in the torso. All spectroscopy tools will use localized techniques, to provide volumetric hepatic metabolite measures. Hepatic energy homeostasis (ATP values) will be measured using an ultra-short TE sequence with "echo times" as short as 70 μs, which increases the signal to noise ratio allowing shorter temporal sampling if needed. Hepatic lipid and water signals will be measured within a breath hold using ^{1}H MRI sequences .

We will adapt the MRS acquisitions to provide a known reference against which to calibrate hepatic metabolite values. By calibrating the metabolites values, we will know exactly which metabolite(s) change and by how much, both within our cohort and in response to our dietary intervention study.

2.6. Summary

This study will advance several goals of the NIH Action Plan: 1) establish a multidisciplinary team to develop quantitative methodologies and imaging protocols for liver, 2) validate diagnostic criteria and methodologies for imaging in liver in both a cross-sectional and a longitudinal dietary intervention study of patients with NAFLD, 3) create a liver tissue bank with correlative imaging data, 4) develop reliable non-invasive MR markers to distinguish simple steatosis from NASH, and 5) define the *dynamic* changes in metabolism, energy homeostasis, and MR biomarkers as they relate to fructose-related liver injury.

3. Study design

3.1 Design overview

The proposed open label study is single-center randomized, phase II clinical trial designed to address the following specific aims:

a) To optimize an MR protocol to characterize the range and rates of *baseline and dynamic* measures of metabolism and energy homeostasis following an acute IV fructose challenge. Volumetric and quantitative measures of hepatic lipid deposition, energy levels, and water homeostasis will be characterized in normal volunteers to determine typical ranges and coefficients of variation for each technique.

b) **To differentiate the baseline and dynamic changes of metabolic and energy homeostasis following an acute IV fructose challenge in patients with biopsy-proven NAFLD**. MR-based measurements from the Aim 1 protocol will be correlated to clinical measures of metabolism, energy homeostasis and histologic disease activity before, during and at 4 and 24 hours following IV fructose challenge in regions co-localized and distant from biopsy.

A cohort study of healthy volunteers (n=30) and patients with NAFLD (n=70) will be enrolled. Healthy volunteers will have no evidence of chronic liver disease (normal liver enzymes and normal liver imaging and/or a liver biopsy performed for clinical evaluation of liver disease but whose pathology results note no pathologic abnormalities). Patients with suspected NAFLD who will undergo a standard-of-care liver biopsy, or who have had a historical biopsy within 6

months of the Screening visit, will have both acute intravenous fructose challenge for assessing baseline and post-dietary intervention *static and dynamic* measures of metabolism and energy homeostasis. Co-localization of liver biopsy with MRI to optimize / assess MRI measures of the histologic features of NAFLD will be performed to develop MRI as a surrogate "biomarker" for liver biopsy. With the exception of the liver biopsy, no accurate non-invasive biomarker for disease activity currently exists.

Screening for eligibility and collection of baseline data will span up to 8 weeks. Patients who have had a liver biopsy showing NAFLD within 6 months of the Screening visit or patients who will be undergoing a standard of care percutaneous liver biopsy for NAFLD will be consented.

Measures of acute changes in metabolism and energy homeostasis will be performed the morning of the liver biopsy. The primary comparisons will be made using an intention-to-treat analysis of the change in the (a) metabolic and (b) energy homeostatic measures from baseline. (c) compartment volumes by MRI, Secondary outcomes measures will include changes in clinical and histologic features of NAFLD as assessed by liver enzymes and the NAFLD Activity Score (NAS), as determined from standardized histologic scoring of liver biopsies. This study will be conducted in compliance with the protocol, Good Clinical Practice (GCP), and all applicable regulatory requirements.

3.2 Population

Participants at least 18 years of age, both healthy controls and those with suspected NAFLD.

3.4 Sample size: 30 healthy controls and 70 subjects with biopsy-proven NAFLD

3.5 Type of trial

Single-center, phase 1 exploratory study to evaluate the acute (baseline and dynamic) changes in metabolic, energy homeostatic and MR biomarkers in volunteers and patients with biopsy-proven NAFLD measures following IV challenge and dietary fructose restriction.

3.6 Outcome measures

Acute Fructose Challenge and MR Biomarkers

Primary: The difference(s) in rate and extent of the dynamic changes in metabolism and energy and water homeostasis in patients with biopsy-proven NAFLD. Liver histology will be the primary predictor of disease and the primary outcome variable to which secondary measures (i.e. MR-biomarkers, metabolic parameters etc) will be measured.

4. MRI Biomarkers of NAFLD

4.1 MR-based Measures of Hepatic Energy Homeostasis

^{31}P Ultra-short TE MRS pulse sequence used in the Duke Center for Advanced MR Development (CAMRD) for the dynamic measurement of phosphorus metabolites is very similar in design to the UTE-MRS method by Cortez-Pinto, et al. (24) just without the localization gradients. With a hard pulse excite of 100 μs, we can acquire an FID signal as short as 70 μs from the hard pulse center as compared with a nominal echo time of 2 ms for the standard Siemens slice-select excitation sequence. This gives 2-3 times the SNR for the short T_2 metabolites in the ^{31}P spectrum.

All study scans will be taken on a 3T Siemens TIM Trio-CT scanner in CAMRD. We will contract with Clinical MR Solutions (Dr. Ralph Hashoian, Brookfield, WI) to set the specifications for a transmit/receive (TX/RX) Helmholtz-style ^{31}P tuned flex-coil to provide superior B1 homogeneity to our current surface coil design. Raw data will be transferred off scanner and analyzed using a suite of MRS processing and fitting software written by Dr. Soher.

Possible Problems and Alternative Strategies – If data quality of the UTE MRS is insufficient to characterize the signal changes observed in volunteers and patients, then we will investigate the use of a single dimension encoding method to attempt to exclude confounding signals from tissues surrounding the liver. If problems arise with the data processing and analysis, the jMRUI program can also be used. An additional small reference phantom mounted on the ^{31}P coil will identify its spatial location on MRI.

5. Intravenous Fructose Challenge with MRS Measures of Energy Homeostasis

IV fructose challenge with MRS measures of energy homeostasis will be performed in the morning after an overnight fast. For all subjects, a vein will be catheterized and a venous blood sample drawn for laboratory evaluation (uric acid, glucose, insulin, lipids, and free fatty acids). A slow infusion of isotonic sodium chloride solution will be started to maintain catheter patency. After baseline MR data sets are obtained, fructose (250 mg/kg of body weight) dissolved in 25 to 50 mL of isotonic sodium chloride solution will be infused rapidly (over 30-60 seconds) through the indwelling venous catheter; further spectra will be collected for 1 hour. The slow saline infusion will be continued until the end of the study. At that time, another venous blood sample will be collected for laboratory evaluation (uric acid, glucose, insulin, lipids, and free fatty acids).

6. Standardized Life-Style and Dietary Research Questionnaires

6.1 Alcohol Use Disorders Identification Test (AUDIT) is a 10-item questionnaire with a simple scoring scale that will be administered during screening visit 1. A 3- item interim drinking history (AUDIT-C) measuring consumption in the past 90 days will be obtained during follow-up visits at 3 and 6 months

6.2 Health Related Quality of Life **(HRQOL; SF-36)** is a 36-item, self-report measure designed to assess the quality of life in patients that will be administered during screening.

6.3 Automated Self-Administered 24 Hour Recall **(ASA24™)** is a 24 hour dietary recall tool which will be utilized for the purpose of assessing average fructose consumption. The National Cancer Institute ASA24™ is web-based, allowing patients to access the web-site at their convenience. It has long been regarded as the best methodology to measure food intakes for dietary surveillance, nutritional epidemiology, clinical research, and intervention research because it provides the highest-quality and least biased dietary data for a single day. This method allows collection of detailed intake and portion sizes, and because the data collection occurs after the consumption, this methodology does not affect what an individual chooses to eat on a given day. The close proximity in time to the intake day minimizes memory and cognitive issues that afflict other methodologies. ASA24™ has been developed to permit unannounced, automated, and self-administered 24HR, enabling the administration of multiple days of recalls in large-scale

epidemiologic studies, surveillance studies, behavioral trials, or clinical research, thus enhancing researchers' ability to assess usual dietary intakes. The format and design of ASA24™ are based on a modified version of the interviewer-administered Automated Multiple Pass Method (AMPM) 24HR developed by the U.S. Department of Agriculture (USDA). ASA24™ consists of a respondent Web site and a Researcher Web site. The Respondent Web site allows study participants to report their intake for the previous day. The Researcher Web site allows researchers to register a study and its participants, set study parameters (e.g., number of recalls, number of attempts per recall, time to complete a recall), manage study logistics, and obtain analysis files. The investigators and study coordinators will review this instruction document completely and also to become familiar with the Respondent Web site in order to effectively and successfully use ASA24™. An overview of the Respondent Web site is available online at http://riskfactor.cancer.gov/tools/instruments/asa24/info.html. The ASA24™ allows for scheduled and non-scheduled responder recall nutritional data collection, would accommodate the number of patients in the proposed study, will allow for detailed analysis of nutritional data, and is free tool to researchers.

6.4 ***NCI Dietary Health Questionnaire*** **(DHQ*Web)** is a web-based version identical in content to the original DHQ. By automating the DHQ and providing it on the Web for public use, researchers have another tool to collect and analyze food frequency questionnaire data. DHQ*Web takes advantage of what automated and electronic questionnaires are able to do -- respondents follow automated skip patterns, are queried to complete all questions before proceeding to the next, navigate within the instrument to correct or modify responses, and can log in at any time to complete the questionnaire, starting where they left off. DHQ*Web provides efficiency with respect to data quality because respondents cannot complete the questionnaire with missing or inconsistent responses. Patient will be asked to complete the DHQ at entry.. An overview of the respondent web site is available online at http://riskfactor.cancer.gov/DHQ/webquest/index.html.

7. Liver Histology

Liver histology is the “gold standard” by which to define disease severity for NAFLD. MRI biomarkers as well as the metabolic and energy homeostatic measures associated with histologic features of NAFLD.

8. Patient Selection

8.1 Recruitment: Thirty healthy controls and 70 patients with NAFLD will be recruited from the Duke Liver Clinics. Eligible patients will be identified and recruited by the principle investigator and/or sub-investigator. Recruitment into the study will be performed without regard to gender or ethnicity as any patient with suspected NAFLD will be considered eligible if all inclusion and no exclusion criteria are met.

8.2 Inclusion criteria

Patients must satisfy all of the following criteria to be eligible for enrollment:

- Age > 18 years as of the initial screening interview and provision of consent
- Healthy control as defined by normal liver aminotransferases AND no evidence of NAFLD on radiologic imaging studies OR liver biopsy (if one had been historically performed for evaluation of suspected liver disease).

OR

- Patients who have had a liver biopsy showing NAFLD within 6 months of the Screening visit or patients who will be undergoing a standard of care percutaneous liver biopsy for NAFLD will be consented. Additionally, anyone with a prior diagnosis of NAFLD or NASH-related cirrhosis (histologically or clinically) may also enroll without having had a recent historical biopsy.

8.3 Exclusion criteria

Patients who satisfy any of the following exclusion criteria will be ineligible for enrollment:

- Current or history of significant alcohol consumption for a period of more than 3 consecutive months within 1 year prior to screening (significant alcohol consumption is defined as more than 20 g/day in females and more than 30 g/day in males, on average)
- Inability to reliably quantify alcohol consumption based upon local study physician judgment
- Prior or planned (during the study period) bariatric surgery
- Uncontrolled diabetes defined as HbA1c 9.5% or higher within 60 days prior to enrollment
- A platelet count below 90,000/mm^3
- Clinical evidence of hepatic decompensation as defined by the presence of any of the following abnormalities:
  - Serum albumin less than 3.2 g/dL
  - INR greater than 1.3
  - Direct bilirubin greater than 2.0 mg/dL
  - History of esophageal varices, ascites or hepatic encephalopathy
- Evidence of other forms of chronic liver disease (with the exception of a prior diagnosis of NAFLD or NASH-related cirrhosis)
  - Hepatitis B as defined by presence of hepatitis B surface antigen (HBsAg)
  - Hepatitis C as defined by presence of hepatitis C virus (HCV) RNA or positive hepatitis C antibody (anti-HCV)
  - Evidence of ongoing autoimmune liver disease as defined by consistent liver histology
  - Primary biliary cirrhosis as defined by the presence of at least 2 of these criteria
    - (i) Biochemical evidence of cholestasis based mainly on alkaline phosphatase elevation
    - (ii) Presence of anti-mitochondrial antibody (AMA)
    - (iii) Histologic evidence of nonsuppurative destructive cholangitis and destruction of interlobular bile ducts
  - Primary sclerosing cholangitis
  - Wilson's disease as defined by ceruloplasmin below the limits of normal and consistent liver histology
  - Alpha-1-antitrypsin(A1AT) deficiency as defined by diagnostic features in liver histology (confirmed by alpha-1 antitrypsin level less than normal; exclusion at the discretion of the study physician)
  - History of hemochromatosis or iron overload as defined by presence of 3+ or 4+ stainable iron on liver biopsy
  - Drug-induced liver disease as defined on the basis of typical exposure and history
  - Known bile duct obstruction

  - Suspected or proven liver cancer
  - Any other type of liver disease other than NASH
- Serum alanine aminotransferase (ALT) greater than 300 U/L
- Serum creatinine of 2.0 mg/dL or greater
- Unstable therapy for components of the metabolic syndrome (ie. recent starting or stopping of insulin sensitizing agent, lipid lowering agent, and/or antioxidant therapy). Recent starting or stopping (for more than 7 days) the use of a thiazolidinedione (pioglitazone or rosiglitazone) 90 days before the entry biopsy or anytime thereafter
- Use of any prescription or over-the-counter medication or herbal remedy that are believed to improve or treat NASH or liver disease or obesity for the 90 days prior to baseline liver biopsy or prior to randomization
  - Patients must not take any other agent to treat NASH except the treatment assigned after randomization.
- Inability to safely obtain a liver biopsy
- Active substance abuse including inhaled or injection drugs in the year prior to screening
- Pregnancy, planned pregnancy, potential for pregnancy and unwillingness to use effective birth control during the trial, breast feeding
- Any other condition which, in the opinion of the investigator, would impede compliance or hinder completion of the study
- A contraindication to MRI examinations
- Extreme claustrophobia
- Weight or girth exceeds the scanner capabilities
- Any condition or circumstance that, in the opinion of the site investigator, would interfere with completion of MR examinations
- Failure to give informed consent

9. Trial protocol—Study Visits and Events

9.1. Visit schedule overview

Healthy controls will ONLY undergo:

- Screening for eligibility for enrollment (1-2 visits over a maximum of 8 weeks)
- Acute intravenous fructose challenge with measure of metabolism and energy homeostasis by magnetic resonance spectroscopy (MRS) (baseline and assessment of change over 60 minutes.
- MRI of the liver

Data collected from healthy controls will be used as reference and optimization values for which study will assess metabolic and energy homeostatic measures in those with NAFLD.

Patients with NAFLD will undergo the following related activities study phases:

- Screening for eligibility for enrollment (1-2 visits over a maximum of 8 weeks)
- Acute intravenous fructose challenge with measure of metabolism and energy homeostatasis by magnetic resonance spectroscopy (MRS) (baseline and assessment of change over 60 minutes. MRI of the liver

- Co-localization of non-invasive MR biomarkers with liver biopsy procedure.

9.2. Screening and Baseline Data Collection

Patients who appear to be eligible after chart review and have completed of standard of care tests and procedures for NAFLD or suspected NAFLD will be invited to undergo screening. Recording of screening data may not start until the patient has signed the consent statement. Screening and baseline data collection procedures will include questionnaires, physical examination, measurement of fasting serum glucose and insulin, routine liver, lipid and metabolic tests, uric acid and urine analysis for pregnancy testing. Standard therapy for NAFLD will be reviewed. Risks, potential benefits and alternatives to study participation will be reviewed in detail.

Patients suspected of having NAFLD, requiring a standard of care biopsy to establish their diagnosis and guide their clinical management, will be approached to participate in this study. Patients with a historical biopsy confirming NAFLD within 6 months of the Screening visit will also be approached to participate in this study.

All participants who sign the consent statement will be registered in the trial database. Patient charts will be reviewed for historical information. Each participant who starts screening will be accounted for at the end of screening, as either screening success (enrolling in the trial) or a screening failure. A screening failure is defined as a participant who signed the consent form, but is found to be ineligible prior to randomization; patients who meet medical eligibility criteria but who refuse enrollment in the trial; and patients whose liver biopsies do not meet entry criteria. Reasons for screening failure will be recorded in the trial database.

The patient will sign the consent at screening visit to obtain any tests and procedures needed to finalize eligibility after chart review and will undergo a history and physical examination to identify other abnormalities and contraindications for participation. Anthropomorphic assessments (body weight [kg], body height [m], body mass index [BMI], waist circumference [cm], hip circumference [cm] and waist-to-hip ratio; vital signs (systolic and diastolic blood pressure, heart rate, respiratory rate, body temperature); and general physical findings, including hepatosplenomegaly, peripheral manifestations of liver disease, ascites, wasting or fetor, will be collected and recorded. Use of anti-NASH, antidiabetic, statin and fibrate medications in the 90 days prior to the biopsy will be obtained and recorded. Laboratory test results that need to be recorded from chart review or obtained as part of screening visit 1 include hepatitis B (HBsAg) and hepatitis C (anti-HCV), antinuclear antibody (ANA), anti-smooth muscle antibody (ASMA), anti-mitochondrial antibody (AMA), ceruloplasmin (if patient is less than 40 years old), A1AT concentration, iron and transferrin saturation, immunoglobulins (IgA, IgG, IGM), fasting glucose, insulin, and free fatty acids; CBC (Hgb, WBC, platelets, MCV, hematocrit), prothrombin time and INR, complete metabolic panel (sodium, potassium, chloride, bicarbonate, calcium, phosphate, BUN, creatinine, uric acid, albumin, total protein), GGT and hepatic panel (ALT, AST, alkaline phosphatase, total bilirubin, direct bilirubin), fasting lipid profile (total cholesterol, triglyceride, LDL, HDL), and hemoglobin A1c (HbA1c). Frequency and amount of alcohol intake will be obtained. Women of childbearing potential must have a negative pregnancy test.

9.3. Visit Day -1

All patients will be provided with a "standard" meal the day prior to the fructose challenge MRI in order to control for dietary composition and calorie intake prior to intravenous fructose challenge. Patients will be NPO after midnight for morning IV fructose MR biomarker measures.

9. 4. Visit Day 0:

Patients with suspected NAFLD, who have not had a biopsy in the prior 6 months, will have a standard of care liver biopsy followed by co-localization of liver biopsy with MR.

Patients will be escorted to the Department of Radiology for baseline MRI/MRS imaging (MRI study protocol and data acquisition sequences detailed in **Appendix A**). Time 0 uric acid, insulin, glucose, lipid panel, and free fatty acids will be obtained. IV Fructose will be administered via heplock and dynamic measures of hepatic ATP and other metabolite measures will be obtained over 1 hour. At that time, another venous blood sample will be collected for laboratory evaluation (uric acid, glucose, insulin, lipids, and free fatty acids).

After acquisition of these late hepatic recover ATP measures, for the patients who have not had a historical biopsy in the 6 months prior to screening, a percutaneous liver biopsy will be performed as standard of care. After standard of care procedures and recovery are completed for the percutaneous liver biopsy, a very limited MRI will be performed to co-localize the site of liver biopsy with the exact site of MRI for modeling of non-invasive MRI biomarkers to features of chronic liver disease as characterized on liver biopsy. Subjects with suspected NAFLD may have two MRI's (one for IV fructose challenge and one for co-localization with liver biopsy site). For subjects with NAFLD, study participation will end after completion of the second MRI.

9.5 Adverse event reporting and Data Safety Monitoring

The investigators and staff will monitor and report adverse events to ensure patient safety. Any unanticipated or adverse events will be reported to IRB and NIH (Sponsor) in compliance with government regulations of event reporting in research studies. Dr. Anna Mae Diehl, Chief of the Division of Gastroenterology and researcher with prior experience in the conduct of IV fructose challenge and MRS will serve as a data-safety monitor is any adverse events arise in the context of this study.

9.5.1. Definitions

Adverse event: An adverse event is any untoward medical occurrence that may present itself during treatment or administration with a pharmaceutical product or clinical procedure and which may or may not have a causal relationship with the treatment. Adverse events include any unanticipated problems involving risks to participants, or breaches of protocol which might entail risk to participants. The term "unanticipated problem" includes both new risks and increased rates of anticipated problems.

Serious adverse event: A serious adverse event (SAE) is an adverse event occurring at any time during the study that results in death, life-threatening adverse drug experience, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant disability/incapacity, or a congenital anomaly/birth defect. Other events may also be considered an SAE if, based on medical judgment, the event jeopardized the patient to the point of requiring medical or surgical intervention to prevent the occurrence of any of the conditions for an SAE listed above.

Unexpected adverse event: An unexpected adverse event is any adverse event with specificity or severity that is not consistent with the risk information in the study protocol, current investigator brochure, or current package insert.

Adverse Events associated with the use of the intervention (i.e. fructose administration or restriction): Any unexpected adverse event for which there is reasonable possibility that the adverse experience may have been caused by the administration of intravenous fructose and/or restriction of dietary fructose.

9.5.2. Monitoring for adverse events

Adverse events will be recorded on study data forms whether or not they are thought to be associated with the study. Adverse events may be discovered during regularly scheduled visits or through unscheduled patient contacts between visits. Summary data on adverse events will be promptly assessed by investigators if they occur. Analyses or listings of adverse events will not be provided to the IRBs; however, adverse events involving unanticipated problems involving risks to participants, or breaches of protocol which might entail risk to participants will be reported to the IRB as soon as possible after they are discovered.

10. Statistical design and analysis

Coefficients of variance (CV) will be used to evaluate reproducibility of MRS/MRI baseline measures. Intra-class correlations (ICC) will be used to measure absolute test-retest baseline measure reliability for the test-retest subjects. ICCs will be calculated with a model that separates subject, voxel, measurement and error variances using an analysis of variance (ANOVA) method. We will include a number of multi-factorial random effects models (66, 67) which use a restricted maximum likelihood (REML) algorithm to prevent negative variance terms. The ANOVA model for metabolite estimates across all subjects will be $M_{ijk} = \mu + S_i + V_{ij} + N_k + e_{ijk}$ where μ is the average across all voxels, S_i = subject, V_{ij} = voxel and N_k = scan random effects, respectively and e_{ijk} is residual error. For ICC in individual subjects, we leave out the S_i term. ICCs for test-retest measures will be based on random-effects analysis of the variance models and will be computed as follows: $ICC = \sigma^2_B / (\sigma^2_B+\sigma^2_W+\sigma^2_e)$ where σ^2_B is generalized between-subject variance of metabolites, σ^2_W is within-subject variance between scans 1 and 2 and σ^2_e is variance due to random noise (68). CVs for test-retest reproducibility will be calculated using $CV = sqrt(\sigma^2_w+\sigma^2_e) / \mu$. Similarly we will obtain CVs from the first 20 subjects in Aim2 and will compare these to the normal population.

Dynamic changes will be characterized in a more *ad hoc* manner. From the metabolite estimates at time t (M_{ijkt}), the following paramerters will be measured for each individual (subscript i): baseline (B_i = mean of M_{jkt}, where t = prior to fructose challenge), range of change from baseline ($D_i=B_i-min(M_{jkt})$ if any), time to maximal change (T_i = time to reach $min(M_{ijkt})$), time to baseline recovery (R_i, if applicable). A 95% confidence interval of baseline will be used to test if the metabolite level is recovered to the baseline. If it does not reach the baseline, we will estimate the baseline based on the assumption of a linear recovery rate. Since metabolite levels will be measured at discrete time points, spline smoothing will be used to fit the curve. These values will be averaged across the cohort of volunteers and simple measures of variance calculated.

10.1 Sample size and Statistical Power Consideration: Assuming 20% change in hepatic ATP and uric acid (i.e. before~ Normal(1.2, σ^2) and after ~ Normal(1, σ^2)), one-side test with alpha = 0.15, the power would be i) if $\sigma^2 = 1$, power =0.523 ii) if $\sigma^2 = 0.8$, power = 0.629, iii) if $\sigma^2 = 0.6$, power = 0.784, iv) if $\sigma^2 = 0.5$, power = 0.875.

10.2 Analytical and Statistical Approach: We will test if the acute fructose challenge alters hepatic ATP in healthy volunteers compared to those with NAFLD and whether changes MR parameters and/or energy homeostasis can predict the histologic severity of NAFLD.

10.3 Management of Missing data: The occurrence of missing data in this trial is expected to be low. We estimate that careful selection of patients during the screening phase and the consent process should result in no more than 10% missing data from patients who drop out before completing the study.

10.4 Justification of sample size: A total of 67 patients will be included to compare the efficacy of acute fructose challenge on the metabolic and energy homeostasis changes in patients with NAFLD.

11.0 Human Subject Issues

11.1 Regulatory Compliance

The study protocol, questionnaires, and consent forms will be submitted to IRB for approval. All study personnel must complete training in the Protection of Human Subjects per NIH guidelines. The proposed study will require eligible patients without regard for gender or ethnicity. The patient must sign the consent to be eligible for the trial. The consent form will describe the purpose of the trial, the procedures to be followed, and the risks and benefits of participation. Copies of the signed consent forms will be given to the patient, and this fact will be documented in the patient's study record.

11.2 Risk to Human Subjects

11.*2.1 Intravenous Fructose Challenge*

Fructose has been recommended as an intravenous energy source during parenteral nutrition for patients with hepatic disease, uncontrolled diabetes mellitus, and in the postoperative state. Fructose is metabolised in the liver where it causes increased lactate formation, high-energy-phosphate depletion, increased uric-acid formation, and inhibition of protein synthesis. This results in increased concentrations of blood-lactate and serum-uric-acid. In hepatic disease fructose infusion may theoretically lead to lactic acidosis.

11.2.2. MRI/MRS

Standard safety precautions: screening; exclusion of potential participants with contraindications to MR. MR is a minimal risk procedure if standard precautions and practice exercised. Standard precautions for MR procedures include: claustrophobia, anxiety, discomfort from lying supine for 30-60 minutes, hearing loss, and heating of metal in the body. Patients with metal will be screened by questionnaire and excluded when the presence of metal is known or suspected. Sources include the presence of a cardiac pacemaker or defibrillator; metal fragments in eyes, skin, body; heart valve replacement, brain clips, venous umbrella; being a sheet-metal worker or welder; aneurysm surgery, intracranial bypass, renal, aortic clips; prosthetic devices such as middle ear, eye, joint or penile implants, joint replacements; hearing aid, neurostimulator,

insulin pump; intrauterine device; shunts/stents, metal mesh/coil implants; metal plate/pin/screws/wires, or any other metal implants; permanent eyeliner, eyebrows.

11.2.3. Patient privacy

It is the investigator's responsibility to conduct the protocol under the current version of Declaration of Helsinki, Good Clinical Practice, and rules of local IRBs. The investigator must ensure that the patient's anonymity be maintained during all aspects of their study participation. Patients will be identified only by an identification code but not by their name, SSN, or hospital medical record number. All laboratory specimens, study forms, reports, and other records that are part of the study data collection materials will be identified by coded number to maintain patient confidentiality. All records will be kept in locked office. All electronic records of study data will be identified by coded number. Clinical information will not be released without written permission of the patient, except as necessary for monitoring by the IRB. Consent procedures and forms, and the communication, transmission and storage of patient data will comply with individual site IRB and NIH requirements for compliance with The Health Insurance Portability and Accountability Act (HIPAA). Investigators will maintain a separate confidential enrollment log which matches identifying codes with the patients' names and addresses (i.e., available only to local clinic staff). All study material will be maintained in strict confidence.

11.2.5 Sample Biobanking

It is anticipated that serum, plasma, DNA, and liver tissue from the participants will be stored for future studies related to NASH and possibly other liver/metabolic diseases in our Duke NAFLD Clinical Database and Biorepository. These samples will be stored in a central repository. Specific IRB approval will be as per specific institutional guidelines addressing the issues such as (a) obtaining a separate informed consent, (b) storage, (c) transportation of the material, (d) who will have access to the material, and (e) what investigations will be conducted.

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Appendix A:

MRI/MRS Protocols for Assessment of Energy Homeostasis and MR Biomarkers for NAFLD

In all cases no patient repositioning will need to occur between data acquisitions for different MR nuclei. Primary measures are indicated in blue and include ^{1}H MRI/MRS for water and fat measures and ^{31}P UTE MRS for fructose measures. In all cases, the multiple acquisitions acquired prior to the injection of fructose will serve as test-retest cases for confirming measurement variance in the presence of pathology. Interleaved measures for experimental hypotheses may be moved or removed in order to ensure sufficient primary measures within the allotted MR time slot.

Only MR Slot 1 and Slot 2 protocols shown below will be used on the healthy volunteers for Aim 1 in order to optimize these protocols. The full MR protocol shown below will be performed for NAFLD subjects.

MR Slot 1 (Time 0)	**MR Slot 2 (Post biopsy)**
^{1}H MRI - anatomical ^{1}H MRI/MRS – water/fat ^{31}P UTE MRS – fructose baseline (multiple acquire) **Fructose injection** ^{31}P UTE MRS – fructose dynamic (multiple acquire) ^{1}H EPSI – water/fat ^{31}P UTE MRS – fructose dynamic (multiple acquire)	**^{1}H MRI - anatomical for biopsy localization** ^{31}P UTE MRS – fructose recovery (multiple acquire) ^{1}H MRI/MRS – water/fat